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This project identified several potentially protective *Brucella* antigens: YajC, GroEL, GroES, SOD, SecD, 18kDa, 16kDa and TolB. Some potential virulence factors have also been identified. Antigens have been purified as fusion proteins and/or native antigens for further characterization. Selected antigens have also been cloned into Baculovirus for antigen characterization and immunization purposes. Several vaccinia/*Brucella* recombinants with selected *Brucella* antigens have been produced and have been tested in mice. Mice are able to respond to these antigens immunologically but protection has not yet been achieved. New recombinants using more effective early/late synthetic promoters and able to express IL-12 are being constructed to overcome this problem. Vaccinia/listeriolysin and Brucella L7/l12 recombinants are being constructed to assess vector efficacy. To assess the true protective abilities of the selected *Brucella* antigens, *B. abortus* vaccine strain RB51 disruption mutants have been constructed and DNA vaccines have been produced. These are being tested in mice for their ability to induce specific immune responses and protection against challenge with virulent *Brucella spp.*

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FOREWORD

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Principal Investigator's Signature

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V. INTRODUCTION.

a. Nature of the problem, background and previous work.

Brucellosis affects millions of people worldwide. Humans contract the disease either by consuming infected foods or by coming in contact with animals shedding the organism. The disease is characterized by an undulant fever, cold sweats and general malaise; any exercise will produce pronounced fatigue. If untreated the disease can last from a few weeks to several years. Serious complications leading to death can occur. Tetracycline is the treatment of choice for infected humans. In severe cases tetracycline treatment is supplemented with streptomycin or rifampicin. Brucellosis affects a variety of animals including swine, cattle, sheep, goats, dogs, and camels. Important species of *Brucella* are *suis, abortus, ovis, melitensis, canis*, and *neotomae* each with certain predilection for a particular animal species.

Humans are susceptible to *B. melitensis, B. suis, B. abortus* and *B. canis* in decreasing order. The disease is endemic in many Middle East countries, Asia, Mexico, Central and parts of South America.

Antibiotic resistant mutants of *Brucella*, including tetracycline, rifampicin and streptomycin resistant ones, are not difficult to produce in the laboratory. Such mutants could be utilized in biological warfare resulting in high morbidity with no adequate treatment. Therefore, prevention of infection through vaccination is desirable.

Currently available vaccines can not be used in humans because of their side effects or lack of effectiveness. The current, most used vaccine for protecting cattle against *B. abortus* is strain 19 and for protecting goats against *B. melitensis* is strain Rev 1. Vaccination with these strains leads to seroconversion complicating diagnosis of the disease. Both of these vaccine strains are pathogenic for human beings. *B. abortus* strain RB5I was developed in our laboratory and has now replaced strain 19 (Federal Register 1996) as the vaccine of choice for bovine Brucellosis. This strain is a stable, rough natural mutant devoid of 0-side chain. The strain is able to induce protective immunity in mice (1) and cattle (2,3) without the induction of antibodies to the 0-side chain (1,2). Transfer of T cells from strain RB5I immunized mice will transfer protective immunity against a virulent challenge (4), similar to the immunity transferred to mice by T cells derived from strain 19 immunized mice (5). This confirms previous indications that immunity to *B. abortus* infection requires a strong CMI component (4,5,6,7,8). The specific antigens, which confer strong protective CMI, have yet to be defined.

Macrophages are the principle cells of residence for *Brucella* in infected animals. Resistance usually depends on the correct interaction between T lymphocytes, specific for particular bacterial antigens, and the macrophage (9,10,11,12,13,14). Activation of macrophages by interferon-gamma (INF-γ) will lead to the destruction of intracellular *Brucella* (4). This further suggests that T cells, particularly those responsible for the production of INF-γ (T helper1-CD4+ response) are of major importance in anti-*Brucella* immunity (4). These observations do not eliminate a concomitant protective role for cytotoxic T cells (Tc, CD8+), since protective immunity can be demonstrated by passive transfer of either Th or Tc cells (8). It is possible that in Brucellosis the major role of the Tc cells in protection is production of INF-γ and not direct cytotoxicity. Considering these observations, it is most likely that *Brucella* proteins involved in protective immunity will preferentially stimulate INF-γ producing T cells which in turn activate macrophages enhancing their brucellacidal capabilities. Therefore, if *Brucella* proteins with such characteristics could be identified, they would likely induce a strong and protective CMI response. They could be used in cloning and expression systems that are able to present these antigens appropriately to the immune system.

b. Purpose of current work.

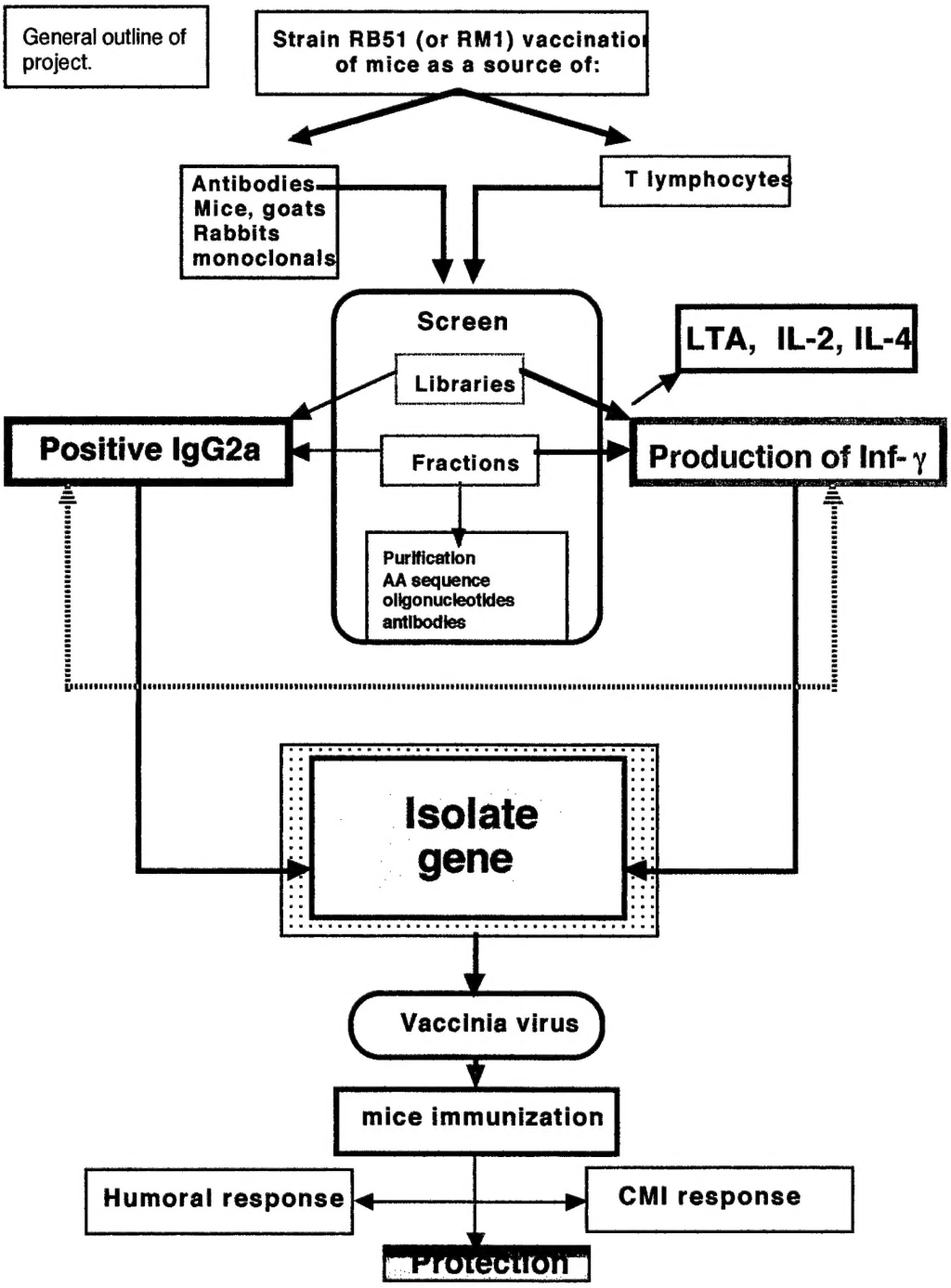
This project intended to identify *Brucella* antigens that are likely to stimulateTh1 responses (with production of INF-γ) in lymphocytes from vaccinated mice and therefore, are likely to have a role in the induction of protective immunity against Brucellosis. Several such antigens have been identified, the genes encoding those antigens were isolated, sequenced and their products characterized to some extent. The genes were used to construct recombinant vaccinia viruses. Vaccinia recombinants were used to immunize mice to determine whether they can induce an immune response and protect mice against a challenge with virulent *B. abortus*. A recombinant vaccinia with protective characteristics against *Brucella* challenge may be a good vaccine candidate for humans.

c. Outline of approach.

The general outline of the approach is found in figure 1. Briefly, mice vaccinated with a protective rough, *Brucella* vaccine (mainly strain RB51) served as source of antibodies to specific *Brucella* antigens and as source of sensitized, reactive lymphocytes . These antibodies and lymphocytes were used to identify reactive *Brucella* antigens. The main approach was to screen *E. coli/Brucella* genomic libraries. In a few instances, *Brucella* antigenic fractions were screened for reactivity. Those clones or fractions reactive with antibodies of the IgG2a type

and/or inducers of lymphocyte proliferation, IL-2 and or Interferon gamma (INF-γ) production were selected as potentially expressing *Brucella* antigens of interest. Once the clones were identified the specific gene(s) were isolated and

Figure1. Outline of project approach.



eventually expressed in vaccinia virus. Antigens identified within the fractions were purified, N-terminal amino acid sequenced and corresponding gene(s) located with the appropriately deduced oligonucleotide probe(s). Vaccinia/Brucella recombinants were tested in mice for their abilty to protect mice against a *Brucella* challenge and their ability to induce an immune response to the recombinant *Brucella* antigen(s).

The approach which gave the best results was screening of the genomic libaries and based on this approach the main methodology used can be outlined as follows:

I. Express *Brucella* antigens in genomic expression libraries and detect potentially protective antigens by either or both:

   1. Reactivity of the recombinant clones with specific antibodies of the IgG2a subisotype found in immunized animals (IgG2a is considered as a potential indicator of Th1 involvement) and

   2. Ability of the recombinant clones to stimulate the lymphocytes from animals immunized with the protective *B.. abortus* strain RB5I vaccine by testing for their ability to proliferate and produce INF-γ upon exposure to such antigens. At later stages of the project we added cytotoxicity assays to detect induction of T cytotoxic (Tc) cells and flowcytometric analysis.

II. Subclone genes identified by the above outlined procedures into vaccinia virus (Western Reserve and/or Wyeth strains). Test the recombinants for their ability to express the antigens *in vitro* and their ability to induce protection in Balb/C mice. Analyze to some extent the character of the immune response induced by the recombinant vaccine and if protective, analyze in detail the immunological parameters associated with protection.

III. In order to obtain unequivocal results for in vitro CMI analysis, antigens have to be purified. Three main approaches were used: a) overexpression of specific genes in *E.coli* as fusion proteins of either thioredoxin (pThio-His expression system, Invitrogen, Inc.) or maltose binding protein (pMal expression system, Novagen) and purification of the recombinant proteins on metal or amylose affinity chromatography. b) ion exchange purification of *Brucella* recombinant proteins from *E.coli* and c) production of the *Brucella* antigens of interest in bacculovirus.

VI. BODY.

A. Detection and characterization of potentially protective antigens.

This project has detected various *Brucella* proteins which could be involved in the protective immune response. Genes of most of these proteins have now been sequenced and characterized. Some of these genes are being or have been cloned into vaccinia for protection and immune response studies.

1. Preparation of antisera and sensitized lymphocytes.
In order to prepare antisera to be used in the detection of antigens goats and mice were immunized as follows:

1. Two Goats were immunized three times with killed (therefore preventing any potential infection), whole or sonicates of *B. abortus* strain RB5I cells emulsified in adjuvant (goats 47 and 48).
2. BALB/C mice were vaccinated with *B. abortus* strain RB5I or *with B. melitensis* strain VTRMI (produced in our laboratory). Groups of 5 to 10 mice were vaccinated once or twice intraperitoneally (ip) with 1 x 10^8 viable organisms and sera obtained 4 weeks after the last immunization were pooled. Preimmunization sera from nonimmunized animals are used as negative controls. Sera were absorbed with whole and sonicated E. coli before use in the screening assays to avoid non-specific background reactions.
3.Sensitized lymphocytes were produced as follows: Splenic lymphocytes obtained from mice 6 to 8 weeks post ip vaccination with strain RB5I were used as a T cell source. Three to 4 mice were immunized weekly when appropriate to keep a continuous supply of lymphocytes available. Preimmunization lymphocytes from nonimmunized animals are used as negative controls.
4. Monoclonal antibodies specific for INF-γ were obtained in the laboratory from hybridoma supernatants and purifying the monoclonal by affinity chromatography. The pure monoclonals were used as the capture antibody in the INF-γ ELISAS.

2. Genomic libraries prepared, screening procedures and antigens selected.

Lambda gtll library.
The lambda gtll library was initially obtained from Dr. R. Warren (WRAIR). Genomic DNA from *Brucella melitensis* I6M was partially digested with HaeIII giving fragments ranging from 15 to 1 Kb, the majority being 2.5 to 3.0 Kb. After precipitation, the DNA was treated with EcoRI methylase followed by attachment of EcoRI linkers. The linkers were then cut with EcoRI allowing subsequent ligation to EcoRI cut Lambda gtll arms. The ligation mixture was packaged *in vitro* and used to infect *E. coli* YIO9O. The lambda gtll expression library was

screened by plaque lifts using goat anti-*B. abortus* RB5I serum (goats 47 & 48). Unfortunately the goat serum had a high background titer to *E. coli* which was very difficult to eliminate even after adsorption of the serum with intact, sonicated, and lambda gtll lysed *E. coli.* To check that the library was expressing proteins detectable using the goat 47 & 48 serum, Western blot analysis and dot blot analysis of the whole library were done. There were several weakly detected proteins in the size range of 45 to 66 kDa that were not seen in either *E. coli* only or *E. coli* infected with native lambda gtll; no fusion proteins were detected. Further immunological screening of the library was done by plaque lift after adsorption of the serum, but most of the putative positive phage were negative on re-screening indicating that the frequency of serologically positive clones was extremely low, consistent with others (23). Lysates of the suspected positive phage were screened along with lysates from pools of different phage recombinants (pools of 10, 50, and 100) using the lymphocyte transformation assay (LTA), all lysates having been standardized for protein content. Once again the relatively low levels of expressed proteins compared to the high background stimulation by *E. coli* in this system proved problematic.

E. coli Cla bank and antigen selection.

Due to the low frequency of positive clones being found in the lambda gtll library we also constructed a *Brucella* gene bank in *E. coli.* Genomic DNA from *Brucella abortus* strain 2308 was partially digested with the restriction enzyme ClaI. DNA fragments of approximately 5Kb were cloned into the ClaI site within the multiple cloning site of plasmid pBBRIMCS. The resulting "Cla bank" was subsequently transformed into *E. coli* strain DH5a and transformants screened to determine the average insert size as well as to confirm a variety of different clones had been generated. Analyzing 2500 clones would screen a genomic equivalent.

Two approaches for screening this library were undertaken:
a)Serological screening and,
b) screening of sensitized lymphocytes using the LTA and INF-g, Il-2 and Il-4 detection systems based on a commercial ELISA kits.

Before doing extensive amounts of work with this library, dot- blot and western blot analysis were performed to check that there were clones present expressing *Brucella* proteins. Dot blot results indicated that the library contained clones that express *Brucella* proteins, however, the size range could not be determined as the reactivity on Western blot was weak. On secondary screening of all putative positive clones, there was a total of 15 IgG positive clones: 9 clones IgG positive with both goat and mouse sera, 4 more that were positive with mouse sera only and 1 more that was positive by goat serum only. All the confirmed mouse positive clones were found to react with mouse antibodies of the IgG2a subisotype indicating potential involvement of a Thl

lymphocyte response with INF-γ production. All the 15, IgG positive clones were tested for their ability to stimulate T cells from sensitized mice to produce INF-γ, an indicator of T cell stimulation but not B cell stimulation. Also, since INF-γ plays a major role in facilitating the elimination of *Brucella* from macrophages, antigens that induce production of INF-γ should have a high probability of being involved in protective immunity. Preliminary data indicated that 11 of the 15 clones stimulate interferon gamma production. All 15 of the clones stimulated lymphocytes to produce a small amount of interleukin-2 (IL-2) but no interleukin-4 (IL-4) indicating that the antigens being selected are most likely Th1 and not Th2 inducers.

The following *Brucella* antigens obtained from the *E.coli* clones were eventually selected for characterization and cloning into vaccinia : YajC, SecD, 18kDa, 16kDa, GroEl, GroES and Cu/Zn SOD. Additional antigens were identified but more work is needed to understand their potential importance. These are: TolB (38% similarity to colicin transport), L15 (17% similarity; ribosomal protein on 50S subunit) and Pal (26% similarity; peptidoglycan associated lipoprotein).

In order to detect additional antigens possibly associated with protection but which may not be detectable by the above outlined screening methods (appearance of specific antibodies or detection of reactive T cells using proliferation or INF-γ detection assays) we have also looked for potential virulence factors of *Brucella*. Immune responses to virulence factors do not have to be necessarily associated with protection but, often protective antigens are virulence factors.
Therefore, isolation, identification and characterization of *Brucella* genes that may be responsible for the intracellular survival and or ability of the parasite to persist in animals appeared to be pertinent to this project.

We prepared a *B. abortus* chromosomal library in pBR322 (sal) and a smaller *B. melitensis* chromosomal library in pBlueScript (HindIII) in *E.coli* and tested them for their ability to survive in mice for 48 hr after intraperitoneal (ip) injection. Approximately 10 to 12 *E. coli* clones were pooled and injected simultaneously into mice in order to accommodate the large number of clones being tested for increased virulence. The *E.coli* strains with just the vectors (pBR322 and pBlueScript) served as controls. It was determined that 10^8 colony forming units (c.f.u) of these control *E.coli* were cleared within 24 hours, when given ip.

Twelve pools of *E. coli* clones from the *B. abortus* library and four pools from the *B. melitensis* library survived for over 48 hours in the spleens of mice after ip inoculations. The vast majority of the pools were cleared from the mice within 48 hours. At this stage we are concentrating our efforts on 4 clones able to

survive in mice for more than 48 hours and which were isolated from the *B. melitensis* library (table 1). The clones were selected by isolating individual colonies on plates and testing for the presence and characterization of their plasmids.

Table1. Preliminary Analysis of Sequencing data for the following four pBSMB clones

Clone # the clone	Insert Size in kb (Fig.1)	Completed Sequence (bp)	Homology
pBSMB31	~6-7	~1939	VACB Protein
pBSMB28F	~5-6	~712	ATP synthase
pBSMB16	~1.6	~1265	Transcriptional Regu
pBSMB12	~2.8	~800	unknown

From the above results, it can be concluded that three clones (pBSMB 31, pBSMB 28, and pBSMB 16) may be associated with intracellular survival or virulence in general.. We will continue to study these clones and genes they contain to assess if they are involved in protection.

c. Immunological characteristics of selected antigens.

Some immunigenic characteristics of the selected antigens, effect of their deletion in vaccine strain RB51 and status regarding antigen purification and cloning into vaccinia are summarized in table2.

As can be observed selected antigens 18kDa, 16kDa, GroEL, GroES, SOD and YajC are involved in the immune response to *Brucella* and represent potential candidates for protective antigens. L7/L12 is a protective antigen described by others and is being included because of this fact. 18kDa deletion mutants of vaccine strain RB51 protect as well as the parent vaccine strain indicating that although the 18kDa antigen has the right in vitro immunological profile (induction of IgG2a antibodies and lymphocyte proliferation with INF-γ production) it may not be a protective antigen. On the other hand, deletion of SOD reduces the protective ability of the vaccine strain suggesting the potential importance of SOD in the induction of a protective immune response.
Very little was known about protein YajC in Brucella and YajC in general. A special effort was made to characterize this protein and its gene.

Table 2
Proteins (Antigens) in use and stages of characterization.

Antigen	IR in Brucella exposed humoral	CMI INF-γ	CMI LTA	ΔRB51	Δeffect on protection	purified	in vaccinia
18kDa	yes	yes	yes	yes	none	pmal	yes
16kDa	(yes)	NED	yes	IP	ND	pmal	IP
GroEL	yes	NED	yes***	Lethal	ND	pmal&thio	yes
GroES	no	yes	ND	Lethal	ND	thio	IP
SOD	no*	yes	weak	yes	decrease	thio,native ionexch	yes
YajC	yes	yes	yes	yes	ND	pmal	IP
SecD	no	NED	NED	IP**	ND	pmal(NC)	ND
TolB	yes@	yes@	yes@	ND	ND	ND	ND
L7/L12	yes	yes#	yes#	Lethal	ND	pmal	IP

NED: Not enough data; IP: in progress; ND: Not done yet, NC: not completed
* = depends on animal species
** = A ΔYajc will also be a Δ SecD ; *** = to be confirmed
@ = Observed with whole clone (may include additional gene products)
# Splitter

YajC/SecD. One selected positive clone (clone *E. coli* MCB 68) contained an insert of 2.6 kb; nucleotide sequence analysis of this insert revealed two open reading frames (ORF). The deduced amino acid sequences of the first and second ORFs had significant similarities with the YajC and SecD proteins, respectively, of several bacterial species. Both the YajC and SecD proteins were expressed in *E. coli* as fusion proteins with maltose binding protein (MBP). In Western blots, sera from mice vaccinated with *B. abortus* strain RB51 recognized YajC but not SecD. Further Western blot analysis with purified recombinant YajC protein indicated that mice inoculated with *B. abortus* strains 19 or 2308, or *B. melitensis* strain RM1 also produced antibodies to YajC. In response to *in vitro* stimulation with recombinant MBP-YajC fusion protein, splenocytes from mice vaccinated with *B. abortus* strain RB51 were able to proliferate and, importantly, produce interferon- gamma (table 3), but not IL-4 indicating a type Th1 response. This demonstrates, for the first time, the involvement of YajC protein in an immune response to an infectious agent. Expression of *B. abortus* YajC and partial SecD proteins was achieved in *E. coli* as maltose binding protein (MBP) fusions.

Table 3. Production of Interferon-γ by splenocytes of RB51vaccinated and naive mice after in vitro stimulation with specific antigens and mitogen.

Stimulant	Interferon-γ (ng/ml) in splenocyte culture supernatants (Mean ± Standard deviation; n = 3)			
	Vaccinated mice		Normal mice	
	3 day cultures	5 day cultures	3 day cultures	5 day cultures
Media	0	0	0	0
ConA	30.55 ± 0.65	27.96 ± 0.43	28.31 ± 2.00	26.05 ± 0.89
RB51	29.63 ± 1.00	29.12 ± 0.30	0	0
MBP-YajC	0.42 ± 0.10	2.90 ± 0.89	0	0
MBP	0	0	0	0

It is interesting to notice nevertheless that the whole, killed RB51 organisms induced highly significant higher levels of INF-j when compared to the purified YajC::MBP.

16kDa protein. Originally, we received pRS44.8 (LSU); this is a recombinant plasmid of pRSET B containing the gene fragment (14 kDa fusion protein). It was thought that this was the gene fragment encoding the carboxyl terminus of a much larger, 60 kDa protein. Instead, our results independently confirmed that the entire gene encodes a much smaller 16 kDa protein. To obtain the amino- terminus of the 16 kDa gene, sets of primer pairs were designed by using Primer Select computer analysis software (DNASTAR, Inc., Madison, WI). The reverse primer, 14kDmid, was taken from known sequence of the carboxyl fragment, pRS44.8. Random primers, named P1-P5, were then used in concert (as forward primers) with 14kDmid. The primer pair P2-14kDmid produced a 518-bp fragment encoding for the amino-terminus; this was confirmed by Southern blot analysis. The PCR fragment was then cloned into pCR II (TA Cloning®, Invitrogen) and sequenced using the dideoxy-mediated chain termination method. This sequence data gave rise to the construction of the primer 16kDstart.

To obtain the full 16 kDa gene sequence, two primers, 16kDstart and 16rev were selected. Primer 16kDstart is a 36 basepair (bp) oligomer containing an internal *Bgl* II site. Primer 16rev is a 34 bp oligomer containing an internal *Kpn* I site. PCR was performed and the 452 bp fragment was subcloned into pCR II. The resulting construct was designated pTA16start and was used to transform *E. coli* DH5α cells.

The entire 16 kDa protein gene is 441 bp (147 amino acids) in length. It contains a ribosomal binding site (RBS) upstream. Computer analysis of the deduced amino acid sequence indicated a signal sequence at the amino

terminus.
Analysis of the nucleotide sequence indicated a potential prokaryotic promoter at 300 bp upstream to the start codon. Currently, we are testing the functional ability of this promoter region in *E. coli* and *B. abortus*.

10.2kDa and 11.6kDa proteins.

Two proteins (10.2 kDa and 11.6 kDa) present in an ammonium sulfate extract of strain RB51(fraction S2) were identified which stimulated production of INF-γ by proliferating lymphocytes from RB51 vaccinated mice. The N-terminal amino acid sequences were determined. Amino acid sequences were back translated using the preferred codon usage of *Brucella* and the generated oligonucleotides probes were digoxigenin labeled. The oligonucleotide sequences used for the generation of probes were:
i.) for the 10.2kDa amino acid sequence :

gccttcgcctaagtggtgggttaccgccatggcctggaagaaggtt

ii) for the 11.6 kDa amino acid sequence:

gcccagccgatgttcatcgttctgctgtagacgttgttggcgtt

Unfortunately both the oligonucleotides probes failed to hybridize to the *Brucella* chromosomal DNA. As an alternative method we are planing to screen our *Brucella* genomic library for the presence of these proteins. We have prepared a specific antisera to these 2 proteins in goat (goat 52) since mice vaccinated with strain RB51 do not produce antibodies to these 2 proteins, although they do develop a good *in vitro* lymphocyte response to them. Goat (52) was immunized with the two specific proteins obtained by cutting the corresponding bands out of SDS-PAGE preparations of strain RB51 fraction S2. The bands were minced in physiological saline and emulsified in Freund's incomplete adjuvant. The goat was immunized subcutaneously 3 times with this preparation. The resulting antisera reacted very strongly with the 10.2 and 11.6 kDa proteins. We have now determinated that the 10.2 kDa protein is GroES therefore, a specific sera for GroES is available.

B. Purification of selected antigens.

As mentioned before, selection of antigens as candidates of protective antigens is based on the ability of T cells from *Brucella* immunized mice to react with the antigens *in vitro* in proliferation assays and induction of INF-γ. In order to insure that the selected antigens are inducing these responses, it is important to have these antigens in pure form that can be achieved by using recombinant protein expression systems. In order to purify the expressed recombinant proteins, a practical route is to express them as fusion proteins. For this purpose we are overexpressing the selected *Brucella* genes in *E.coli* as fusion proteins of either thioredoxin (pThio-His expression system) or maltose binding protein (pMal expression system) and purification of the recombinant proteins on metal

or amylose affinity chromatography. This was done with commercial kits and modifications were introduced to optimize protein yield (into several mgs) and purity. Quantification of purified fusion proteins is carried out using BioRad microtiter protein assays and purity is assessed by Western blot analysis. We have now good quantities (in mgs range) of the following fusion proteins available for continuous testing:
GroES::Thio-his, YajC::MBP, Cu/Zn SOD::Thio-his, Lysteriolysin::Thio-his and GroEL::MBP. The GroEL::MBP is missing approximately 30 aminoacids at the C terminal end of the GroEL. Because of this problem we are also producing a GroEL::Thio-his. Future work will use this GroEl fusion protein instead of the GroEL::MBP.

The complete genes of proteins L7/L12 and the 16 kDa antigens were obtained by PCR amplification. These antigens were also overexpressed as MBP fusion proteins in *E. coli* using the pMalC2 vector system. During the purification process it was observed that the MBP-L7/L12 fusion protein had low affinity to the amylose resin, leading to low quantities of purified protein. Hence, the MBP-L7/L12 protein has been purified from the *E. coli* extracts on an anion exchange resin (HiTrapQ) followed by size exclusion chromatography on Sephadex G-75.
The level of expression of the MBP-16 kDa fusion protein was much lower than the other fusion proteins. This could be because of the presence of a hydrophobic signal sequence at the amino terminus of the 16 kDa antigen. Presently, we are trying to increase the expression levels by removing the signal sequence.
The Cu/ZnSOD was also purified as a non-fusion native protein from *E. coli*. For this, the SOD gene along with its own promoter was cloned into a high copy number plasmid, pBluescript SK-. *E. coli* containing this plasmid expressed high levels of SOD. This SOD was purified by a Triton-X100 treatment of the *E. coli* cells followed by an anion exchange chromatography on a HiTrapQ column. The Triton-X100 treatment released mostly the periplasmic proteins of E. coli of which the SOD comprised a major proportion. On the anion exchange column all the proteins except SOD bound to the column. This method of purification resulted in high quantities of purified SOD in its native form.

Purification of recombinant fusion proteins from *E. coli* even by affinity chromatography can not always guarantee 100% purity. This is a problem if impurities have biological effects on our *in vitro* immune response testing system. Also, the fusion partner may influence the *in vitro* response. In order to have an additional antigen purification method that would not utilize fusion proteins, we selected the expression of antigens in Baculovirus (BV). Also, *in vitro* recombinant BV production systems utilize components (insect cells lines, insect virus) which are unlikely to stimulate non-specific reactions in our *in vitro* testing systems.

The BAC-TO-BAC Baculovirus expression system of GibcoBRL (Grand Island, NY) was used to yield Baculovirus recombinants. It is based on site-specific transposition of an expression cassette into a BV shuttle vector (bacmid) propagated in *E. coli.* Genes to be expressed are inserted into the multiple cloning site of pFASTBAC1 downstream from the polyhedrin promoter.

B. abortus-pFASTBAC1 donor plasmids were generated for the 60 kDa molecular chaperon GroEL, the 10 kDa GroES and the 54 kDa heatshock protein HtrA. The identity of the cloned *B. abortus* genes were demonstrated by hybridization of digoxygenin-labeled DNAs to Southern blotted restriction enzyme digests of the pFASTBAC1 donor plasmids and recombinant bacmids.

Spodopternfrugiperda (Sf9) cells were transfected with the recombinant bacmid DNA using CELLFECTIN reagent. Recombinant virus was harvested at 72 hrs. To analyze the protein expression, large volume of cultures was infected with recombinant viruses and the cells were harvested at 48 hours post-infection. Samples of lysed and infected cells were analyzed on 10 % or 15 % SD S-PAGE gels followed by Western blotting. Western membranes were incubated with polyclonal goat anti-B. *abortus* , polyclonal goat anti-GroES (Goat 52), monoclonal mouse anti-GroEL, or monoclonal mouse anti-HtrA antibodies. Bound antibodies were detected with goat anti-mouse IgG or rabbit anti-goat IgG, conjugated to horseradish peroxidase, using 4-choloro- 1 -naphthol. Recombinant viruses were titrated using *Sf9* cells and the titers were calculated by the method of Reed and Muench and expressed in median infective dose per ml (TCID50/ml)). We have used the Sf9 cells infected with *Brucella* antigen expressing BV as antigens for *in vitro* lymphocyte transformation assay using lymphocytes from non-immunized normal mice and have determinated that these preparations will not cause non-specific stimulations of lymphocytes. This opens the possibility that the *Brucella* expressing BV can be used as specific antigens in our *in vitro* systems. Preliminary mouse immunization experiments with the recombinant BV indicate that these viruses can induce specific immune responses to the *Brucella* proteins.

C. Cloning into Vaccinia virus.

1. Characteristics of Early vaccinia/Brucella recombinants expressing *Brucella* HtrA, GroEL or Cu/Zn SOD.

Crucial to the success of this proposal was to demonstrate that vaccinia virus is able to express *Brucella* proteins and that animals vaccinated with these recombinants are able to recognize the expressed *Brucella* proteins. To test this point we cloned into vaccinia virus Western reserve (WR) strain the gene coding for the *Brucella* Cu/Zn SOD (recombinant WRpUBSOD) and the gene coding for the *Brucella* High temperature requirement A (HtrA) protein (recombinant WRpSBHtrA). We selected these 2 genes because of our previous experience

with these proteins and the availability the DNA sequences in our laboratory.

The results of this study clearly indicated that vaccinia virus can express *Brucella* proteins using our methodology since both proteins, Cu/Zn SOD and HtrA were expressed *in vitro*. We also demonstrated that this expression occurs *in vivo* since the mice responded immunologically to the HtrA protein. Mice did not respond immunologically (at least not with a humoral immune response) to the Cu/Zn SOD protein. This findings is not surprising since mice do not respond to this protein even when immunized with *Brucella* probably because of the similarity which exists between *Brucella* and the mouse Cu/Zn SOD (24). In addition to the production of vaccinia recombinants expressing *Brucella* proteins mentioned above we have constructed a GroEl/ vaccinia recombinant which expresses the whole GroEl protein using the Wyeth strain (WySBGroEL) and a protection study was completed. The following vaccinia/ *Brucella* recombinants were used in this ptotection study:

BALB/c mice were vaccinated once with either vaccinia recombinants WySBGroEL, WRpSBHtrA and WRpUBSOD and challenged with virulent *B. abortus* strain 2308 seven weeks later or vaccinated twice 3 weeks apart and challenged four weeks after the second vaccination. Mice were killed at 1 week post challenge and colony forming units (CFU) of *Brucella* per spleen enumerated and expressed as log10 values. Animals were bled before vaccination and at various intervals thereafter. The dose of vaccination was 10^7 median tissue culture infective doses (TCID50) recombinant viruses in 0.1 ml PBS intraperitoneally.

WySBGroEL recombinant did not replicate in mice based on lack of antibody production against both, vaccinia proteins and the *Brucella* GroEL. This observation corresponds with those of other workers concerning recombinants developed with the Wyeth strain. The other 2 recombinants did replicate in mice as reported previously (24) and produced antibodies to vaccinia proteins. The HtrA recombinants induced antibodies to *Brucella* HtrA but the SOD recombinant did not produce antibodies to the *Brucella* CuZn SOD. None of the recombinants protected against challenge with virulent *Brucella* while vaccination with*B. abortus* vaccine strain RB51 induced significant protection (Table 4). The lack of protection by the GroEL recombinant was not unexpected since it did not replicate in mice and therefore the Wyeth strain should not be used vaccinia. Two vaccinations with the recombinant expressing HtrA enhanced infection with the challenge strain suggesting that the HtrA antigen may have a deleterious effect on the protective immune response to *Brucella*. The data also indicate that two inoculations with vaccinia, 3 weeks apart will induce some level of non-specific immunity to *Brucella* (see statistically significant difference among groups 3, once vaccinated or twice vaccinated). Data also confirms previous non published observations that two immunizations with RB51 lead to higher protection levels than 1 vaccination.

Table 4.Vaccination with Vaccinia/Brucella recombinants. Results

1 vaccination

Group	Vaccination day 0	Challenge day 49	Serology at challenge antibodies to:	log spleen day 56
1	psbHtrA	2308	Vaccinia & HtrA	5.09*
2	psbGroEL	2308	negative	5.33*
3	psc11	2308	Vaccinia only	5.57*
4	pUBSOD	2308	Vaccinia only	5.44*
5	RB51	2308	various Brucella antigens	3.62

* No statistical differences

Psc11: Vaccinia recombinant not expressing *Brucella* antigens (negative control)

2 vaccinations

Group	vaccination day 0 & 21	challenge day 50	serology at challenge antibodies to:	log spleen day 57
1	psbHtrA	2308	Vaccinia & HtrA	5.77**
2	psbGroEL	2308	negative	4.96
3	psc11	2308	Vaccinia only	4.37
4	RB51	2308	various Brucella antigens	2.47

** statistically different from groups 3 and 4.

Group 3, 1 vaccination different from group 3, 2 vaccinations $p = 0.0477$

Analysis of the immune response to *Brucella* antigens induced by the other 2 recombinant vaccines (WRpSBHtrA and WRpUBSOD) indicated a strong anti-

HtrA antibody response after one vaccination but did not reveal anti SOD antibodies. The anti HtrA antibody response is not expected to be protective since passive transfer of anti HtrA antibodies does not confer protection. Potentially, a CMI response to either HtrA or SOD could be protective since T cell transfer experiment with RB51 sensitized T cells is protective in mice. Therefore, we tested recombinant vaccinated mice for a CMI responses *in vitro* using lymphocyte transformation assays (LTA) and INF-γ production and *in vivo* using foot-pad DH reactions.
Mice vaccinated once with the HtrA and SOD vaccinia recombinants and the appropriate controls were tested at 7, 11 and 13 weeks post immunization using the corresponding purified antigens. We were unable to demonstrate a definitive *in vivo* or *in vitro* CMI response in any of the vaccinia vaccinated mice. These data suggest that the present constructs may not be inducing CMI against either HtrA or SOD that may explain the lack of protection. We believe that the failure to induce a strong and detectable CMI response to the *Brucella* antigens by the vaccinia recombinants may be due to the type of promoter we have used in our constructs i.e they do not lead to high expression of the recombinant proteins.

2. WR vaccinia/*Brucella* GoEL recombinant.

Since we discovered that the WySBGroEL recombinant would not replicate in mice new constructs were made with the the WR vaccinia using an early/late vaccinia promoter p7.5 (fig 2).

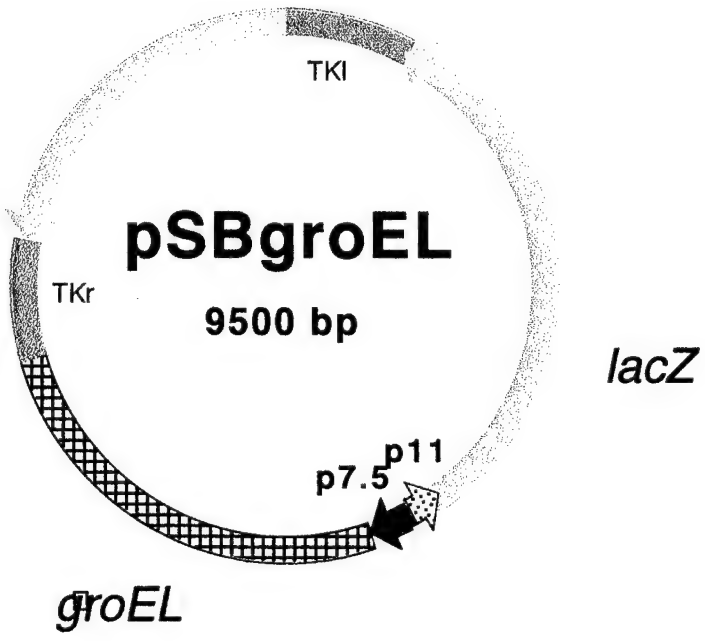


Fig 2. Diagram of recombinant plasmid pSBGroEL. A 1.7 kb fragment containing the *B. abortus groEL* gene (cross hatched line) was cloned into the

shuttle vector pSC11 (light gray regions). The early/late vaccinia virus promoter p7.5 (solid arrow) regulates expression of the *groEL gene* and a late vaccinia virus promoter p11 (dotted arrow) regulates the expression of the *lacZ* gene. The vaccinia virus thymidine kinase sequences (TKl, TKr) flank the expression cassette controlled by the p7.5 and p11 promoters.

This recombinant, denominated WRSBGroEL, was tested for its ability to induce specific antibodies and selected cell mediated immune responses in mice as well as being able to protect these animals against challenge.

Construction of WRSBGroEL (similar strategies have been followed with some modification for the production of additional vaccinia/*Brucella* recombinants described below).
In order to prepare the vaccinia virus expression vector coexpressing *B. abortus* GroEL heat shock protein, plasmid pBA2168 was digested with restriction enzymes *EcoR* I, *Sal* I and *Dra* II . Overhanging ends were filled in and the resulting 1.7 kb fragment containing the *B. abortus gro*EL gene was purified from 1 % agarose gel , the fragment was ligated into the *Sma* I site of shuttle plasmid pSC11 to produce plasmid pSBGroEL. Human thymidine kinase deficient 143B cells (HuTK$^-$ cells) were grown to 80% confluency in Eagle's Minimum Essential Medium (EMEM) containing 5% fetal bovine serum (FBS) in 25 cm^2 flasks, infected with vaccinia virus strain Western Reserve (WR) (ATCC) at a multiplicity of infection (MOI) of 0.05 and incubated. One μg of pSBGroEL was dissolved together with lipofectin, incubated for 25 minutes at room temperature and the mixture (100μl) was added to 1 ml of EMEM and added to WR infected HuTK$^-$ cells at 80% confluency. After a 4+ cytopathic effect (CPE) had developed , the cells were ruptured by 3 cycles of freezing in liquid nitrogen and thawing at 37^0 C. The cell lysates, containing the putative recombinant virions, were serially diluted in 10-fold steps and subcultured onto a new monolayer of HuTK$^-$ cells in flat-bottom six-well plate with EMEM containing 25 μg of bromodeoxyuridine (BdUR) per ml for selection of recombinant virus. Following a 4+ CPE development, the medium was aspirated and the infected cells were overlaid with 1 ml of plaquing media (2x EMEM with 50μg BdUR) containing 0.6 mg/ml of Bluo-gal. Blue plaques, produced by replicating recombinant virions expressing the *lacZ* gene, were collected and used to enhance the virus content of the plaques by inoculating a confluent layer of HuTK$^-$ cells in either 25 cm^2 flasks or six well tissue culture plates. CPE and the presence of blue plaques in the cell monolayer assessed replication of the recombinant virus. Recombinant virus was harvested, plaque purified and enhanced by infecting larger volumes of cell monolayers two more times to develop the recombinant virus WRSBGroEL. Same experiments were also carried out for to generate the vaccinia virus recombinant WRSC11 (control) with shuttle vector pSC11.

Recombinant virus was tested for infectivity, purity and ability to induce GroEL antibodies in mice. 10^4 plaque forming units (pfu) of recombinant virus were injected into each of three BALB/C mice. One of the mice was killed six days later and the recombinant virus was isolated from ovaries and tested for CPE and β-galactosidase activity in HuTK$^-$ cells. Mouse was confirmed infected. The second mouse was killed on day thirteen post infection and the same procedures were followed in order to isolate and to test the infectivity of the recombinant virus in the cell culture. The third mouse was kept alive to obtain serum for serological tests. After isolating the virus from the second mouse, the recombinant virus was plaque purified again and enhanced three more times in order to reach 10^7pfu/ml, the desired infective dose for mice vaccination. Recombinant expression of Brucella GroEL was confirmed by western blotting using anti- B. abortus strain RB51 goat serum (fig. 3).

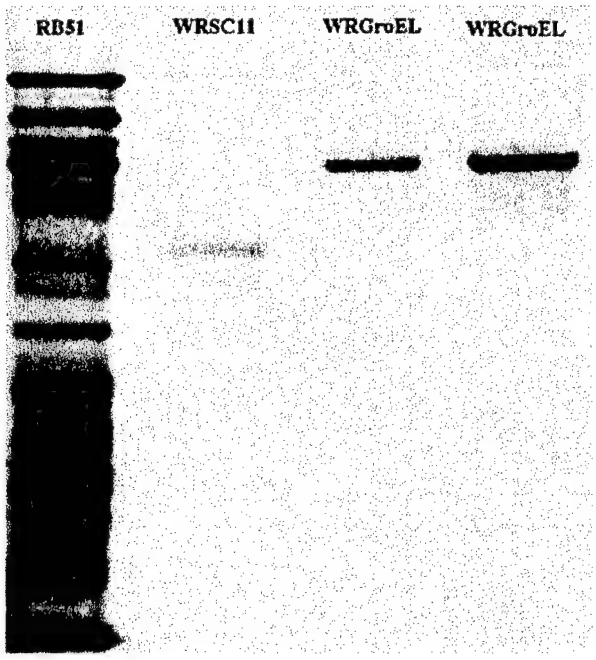


Fig. 3. Western blot analysis of vaccinia virus recombinant WRGroEL (2 different stocks) and plasmid control (WRSC11) and *B. abortus* strain RB51 antigens with goat anti-RB51 serum

Immunization for protection studies was carried out as follows: Five groups of 8 female BALB/C mice each were used. The first and second groups were injected with 10^7pfu of WRSBGroEL intradermally (id) and intraperitoneally (ip) respectively. The third group was inoculated ip with 10^7pfu/ml of vaccinia virus/shuttle plasmid recombinant WRSC11. The fourth group received 2.6 x 10^8 colony forming units (cfu) of *B. abortus* rough strain RB51 (ip) as a positive control for protection and the fifth group received saline ip. Five mice out of each group were challenged with virulent *B. abortus* 8 weeks post immunization and

killed 2 weeks later to assess the number of colony forming units (CFU) in the spleen. The remaining mice were used for serology and in vitro lymphocyte proliferation using various antigens including GroEL::MBP as the specific antigen. GroEL:MBP was missing the last 30 carboxyl end aminoacids of GroEL.

Results can be summarized as follows: Mice vaccinated with the WRSBGroEL developed IgG class antibodies specific for *Brucella* GroEL, lymphocyte proliferation assays results were not conclusive and protection was not observed. Further studies are underway to understand why protection was not induced. The lack of *in vitro* lymphocyte stimulation may be related to the use of GroEL::MBP as the *in vitro* stimulating antigen, as mentioned above the GroEL was missing the last 30 aminoacids and it is possible that the stimulatory epitopes are concentrated in that area. The detailed work with this recombinant is available in a recently MS thesis (Assessment of the expression of *Brucella abortus* heat shock protein, GroEL, in vaccinia virus to induce protection against a Brucella challenge in BALB/c mice. Simge Bologlu, Master of Sciences, Virginia Tech, June 1997).

3. WR vaccinia/*Brucella* recombinants expressing the 18kDa antigen using synthetic early/late promoters.

In general, we now believe that the nature of the promoter (p7.5) used in the previous constructions does not lead to the appropriate *in vivo* expression levels of GroEL needed to induce an appropriate CMI response and therefore, no protection is observed. This possibility had to be explored before more complex issues like appropriateness of GroEL presentation when delivered by vaccinia are investigated. For this reason, new vaccinia recombinant constructs were carried out using a synthetic early/late promoter.

As mentioned above, the lack of a strong specific CMI response in mice vaccinated with the recombinant vaccinia viruses could be due to the nature of the promoters present on the cloning vectors used in the preparation of the recombinant viruses. Recently, shuttle vectors (pMCO2 and pSC65) with a synthetic early/late promoter were developed. Glucoronidase is the marker enzyme in pMCO2 vector whereas in pSC65 it is β-galactosidase (fig. 4). Expression of the cloned genes by the recombinant vaccinia viruses prepared with these new vectors is 1000 times over natural early or late vaccinia promoters.

Higher levels of *Brucella* protein expression by the recombinant vaccinia viruses should enhance the specific and hopefully appropriate CMI responses in the vaccinated mice leading to a better chance of protection against a virulent *Brucella* strain challenge.

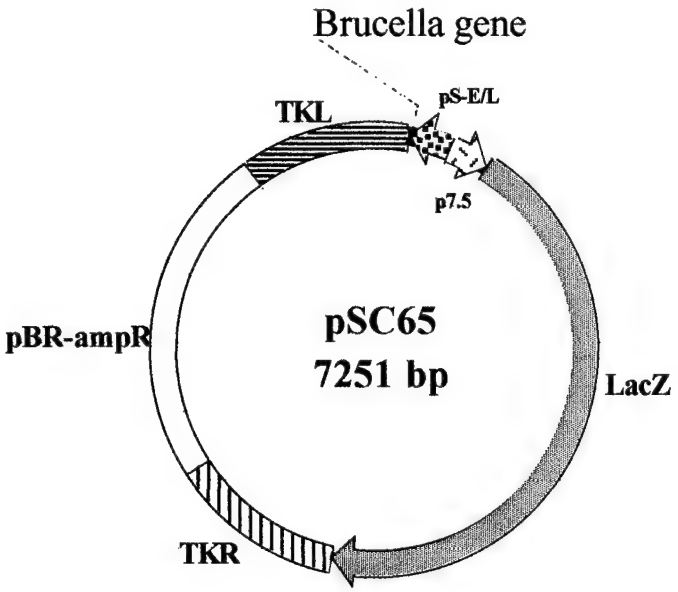


Fig. 4. Schematic representation of the vaccinia shuttle vector pSC65 where *Brucella* genes can be cloned under a synthetic early/late promoter.
Note: pMCO2 contains glucoronidase as the marker enzyme, whereas pSC65 contains β-galactosidase.

Based on this rationale, we cloned the 18kDa gene in shuttle vector pMCO2 and recombinant vaccinia virus was constructed using the WR strain (vWRMC18). Mice were vaccinated ip 10^7 pfu/mouse with vWRMC18 and bled at several time intervals post vaccination. Strong antibody responses were observed against the 18kDa antigen and the vaccinia virus antigens confirming replication of the virus as well as *Brucella* antigen recognition (fig. 5).

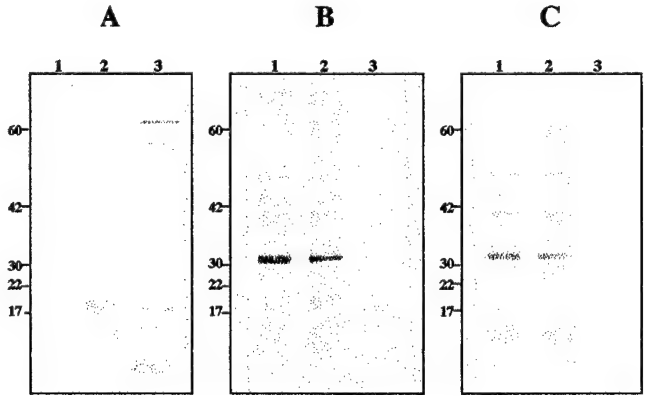


Fig. 5. Extracts from cells infected with the recombinant vaccinia virus containing the 18 kDa protein gene either in the wrong orientation (lane 1) or in the right orientation (lane 2), and *B. abortus* strain RB51 (lane 3) were reacted

with sera from mice vaccinated with *B. abortus* strain RB51 (panel A), the recombinant vaccinia virus expressing the 18 kDa protein (panel B), and the recombinant vaccinia virus not expressing the 18 kDa protein (panel C).

An analysis of the immunoglobulin isotypes involved in the humoral immune response against the 18kDa protein revealed that both, vWRMC18 immunized mice and RB51 immunized mice produced IgG2a antibodies against the 18kDa protein (table 5).

Table 5

Vaccine	ELISA reading against 18kDa antigen (mean +/-SD) IgG	IgG1	IgG2a
vWR18kDa	1.16+/- 0.44	0.67+/- 0.02	0.74+/- 0.25
VWR	0.08+/- 0.06	0.01+/- 0.004	0.01+/- 0.01
RB51	1.91+/- 0.26	0.002+/- 0.001	1.35+/- 0.21

Preliminary flowcytometric analysis of whole blood lymphocyte from vaccinated mice indicate that that the CD4+ cells are responding to the specific 18kDa protein but not the CD8+ cells as indicated by the number of CD69 expressing cells while the total number of CD4+ and CD8+ remains constant (tables 6 and 7).

Lymphocytes obtained from the spleens of these mice proliferated *in vitro* upon stimulation with purified MBP-18 kDa fusion protein and secreted IFN-γ into the culture supernatants. The *in vitro* CMI results combined with the strong antibody responses to 18 kDa, indicate that the synthetic promoter is achieving better results than the promoters used before and encouraged the use of the promoter in future constructions.

The lack of protection in spite of the development of strong humoral and cell mediated immune responses to the 18kDa protein could therefore be related to the following two reasons among others:
a) the 18 kDa antigen is not at all involved in the induction of protection against *Brucella* infection or
b) the recombinant vaccinia virus is unable to express *Brucella* antigens in a way leading to correct processing an induction of protective CMI.

To test the first possibility, we constructed a 18kDa-disruption mutant of vaccine strain RB51 by inserting a kanamycin cassette into the 5' end of the 18kDa gene. Mouse protection experiments performed with this mutant RB51 strain, showed similar level of protection as those obtained with the vaccine strain

strongly suggesting that the 18 kDa antigen is not involved in the elicitation of a protective immune response. This is probably the most likely explanation for the protective failure of vWRMC18. The protection results obtained from this experiment emphasize the importance of using antigens of proven protective capabilities for the construction of the vaccinia recombinants. Therefore, we are now also constructing RB51 disruption mutants using those antigens selected for cloning into vaccinia to asses if they play a significant role in protection. Obviously, no disruption mutants can be obtained with genes that are essential to *Brucella* survival (*groEL, groES*).

Table 6. Percentages of CD69-expressing cells in CD4+ and CD8+ T cells, in peripheral blood, 4 weeks after vaccination. Cells were activated with 3.6 ug/mL 18 KDa protein for 8 hr.

	CD4	CD8
Vaccinia 18 KDa	3.2	0.5
Vaccinia	1.4	0.4
RB51	3.2	0.5
PMA+Iono m.	27	0.8

Table 7. Percentages of Total CD4+ and CD8+ T cells, in peripheral blood, 4 weeks after vaccination.

	Total CD4	Total CD8
Vaccinia 18 KDa	25	7.5
Vaccinia	20.9	6.6
RB51	23.5	4.5
PMA+Iono m.	ND	ND

To test the second possibility (the recombinant vaccinia virus is unable to express *Brucella* antigens in a way leading to correct processing an induction of protective CMI), we will be testing the effectiveness of our vaccinia delivery system by constructing a recombinant virus expressing the listeriolysin protein of *L. monocytogenes.* Listeriolysin is a well demonstrated protective antigen in murine listerial infections (20). The gene for listeriolysin was PCR amplified from the genomic DNA of *L. monocytogenes* strain EGD. Primers for the

amplification was designed based on the published sequence information (21). The part of the gene coding for the signal sequence was not included for the amplification. The amplified fragment was cloned in vaccinia shuttle vector pSC65 and recombinant virus was prepared using WR strain. Upon an initial screening, several recombinant viral plaques expressing the marker enzyme β-galactosidase were obtained. Clear expression of listeriolysin by these recombinant viruses is yet to be demonstrated. The use of recombinant vaccinia virus expressing listeriolysin in mice should demonstrate protection against challenge with *L. monocytogenes* and serve as a positive control for our vaccinia delivery system. Failure to protect mice against *L. monocytogenes* challenge by this recombinant would indicate that our vaccinia system is not an appropriate delivery system for protective *Brucella* antigens and that further research has to be carried out on the expression system itself before a successful vaccine can be engineered.

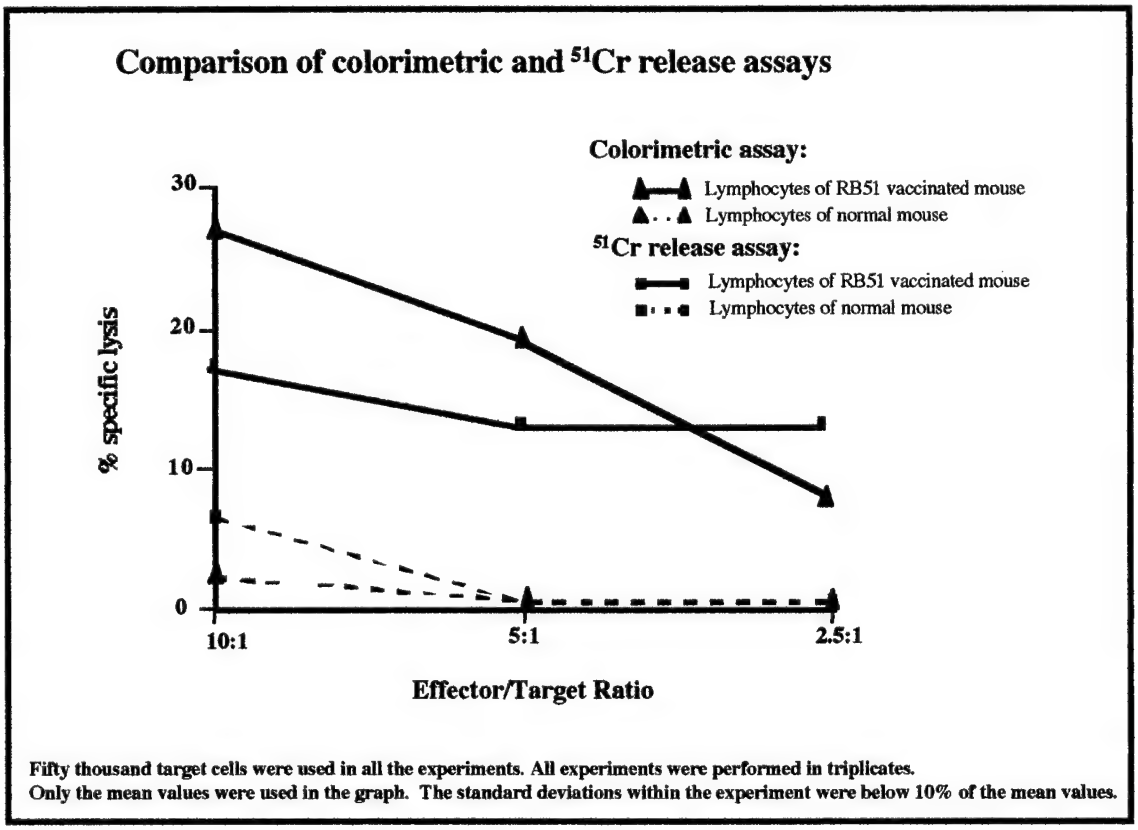
4. Development of a cytotoxicity assay.

Production of INFγ is considered to be one of several crucial immunological events necessary to obtain protective immunity against *Brucella*. However, when challenged with the virulent *B. abortus* strain 2308, these mice did not show any level of protection. Although the immunization with vaccinia/18kDa protein did induce an *in vitro* INFγ response to stimulation with 18kDa::MBP, one can not assume that protection must be observed. Other immunological events may have to occur simultaneously, like the production of specific cytotocic T cells, to obtain protective CMI. We have now standarized cytotoxity assay in the laboratory and are testing for the presence of *Brucella* specific cytotoxic lymphocytes in recombinant vaccinia immunized mice. Establishment and standardization of this test using 51Cr and./ or neutral red release from mouse macrophage cells lines (targets) took several months of work. A detailed description of the test follows and figure 6 compares de 51Cr release assay to the neutral red assay. Stepwise description of test:

1. Infect J774 with Brucella (RB51) usually 100 RB51: 1 Macrophage, incubate overnight (about 12 hrs) in a flask.
2. Wash with gentamycin containing media (add media with 50ug/ml to flask, leave mixing slowly for 5 minutes), remove cells and centrifuge. Resuspend in media with 50 ug gentamycin.
3. Do viable count.
4. Add to 96 well plate (round bottom) 50,000 macrophages per well. Ready to use. Also prepare plates with uninfected macrophages.
5. Get splenocytes from normal and immunized mice.
6. Separate on nylon wool columns (get "fall through" cells mostly T cells).
7. Do viable count
8. Add different proportion of T cells: 12:1 (T cell : Macrophages), 6:1, 3:1, 1.5:1.

9. Incubate overnight (12 hrs not less!).
10. Remove the media.
11. Add neutral red (0.036% in PBS) 200ul.
12. Incubate for 45 minutes
13. Remove the solution and wash cells with PBS very careful so you don't detach or damage cells (3 times).
14. Lyse cells with acetic acid 0.5M + 10% SDS (200ul/well). Pipette up and down several times.
15. Add solution to flat bottom ELISA plate (for reading in machine, round bottom not very good).

Fig 6. Comparison of the 51Cr and neutral red cytotoxicity assays.



5. <u>WR vaccinia/*Brucella* recombinants expressing Brucella Cu/ZN SOD using synthetic early/late promoters.</u>

Since the 18kDa protein role in protection is probably unimportant the question whether higher levels of *Brucella* protein expression by the recombinant vaccinia virus would enhance the specific and hopefully appropriate CMI responses in the vaccinated mice leading to a better chance of protection

against a virulent *Brucella* strain challenge could not be resolved. Therefore, Vaccinia/Brucella SOD recombinants using pMCO2 were made since there is at least circumstantial evidence that SOD is a protective antigen.

The Cu/Zn SOD gene of *Brucella* was amplified by PCR using a custom made primer-pair. Two different restriction enzyme sites were engineered into the primers to facilitate directional cloning of the PCR products. The amplified gene was cloned into the shuttle vector, pMCO2, under the synthetic early/late promoter. Using this vector, the recombinant vaccinia virus (wild type: WR strain) expressing the Cu/Zn SOD was constructed following the established protocols of our laboratory. The recombinant virus was enhanced to a titer of 10^9 pfu/ml. The expression of the SOD protein was confirmed by a Western blot analysis using SOD-specific goat antiserum available in our laboratory.

Mice vaccinated, intraperitoneally, with 10^7 pfu of recombinant virus did not produce any detectable antibody response to SOD and were not protected from the challenge infection with 2 X 10^4 cfu of *B. abortus* strain 2308. Mice vaccinated with strain RB51 do not produce antibodies to Cu/Zn SOD, although they show a weak CMI response to this antigen. Our deletion experiments indicate that the absence of Cu/Zn SOD expression reduces the vaccine efficacy of strain RB51, thereby implying a potential protective role for this antigen. Accordingly, certain peptide sequences of this antigen along with an appropriate adjuvant had been shown to induce a protective response in mice The lack of protective response in mice vaccinated with the recombinant vaccinia virus could be because of insufficient CMI responses to Cu/Zn SOD. Currently, we are analyzing the CMI responses of vaccinated mice to Cu/Zn SOD. Nevertheless, preliminary analysis of the results would indicate that level of *Brucella* antigen expression by the vaccinia recombinant although, probably important, is not the sole factor necessary to induce an appropriate protective immune response.

6. WR vaccinia/*Brucella* recombinants expressing the 16 kDa antigen using synthetic early/late promoters.

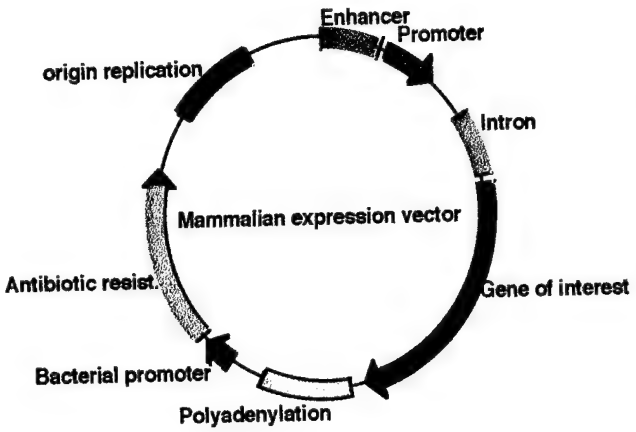
To further explore the question of expression level and induction of immune responses, the full length gene for the 16 kDa antigen was amplified by PCR and cloned into the shuttle vector, pSC65. Construction of the recombinant virus is currently under progress. Several recombinant viral plaques are being subjected to three rounds of plaque purification to select a stable recombinant virus.

D. DNA vaccines.

We are additionally testing the protective ability of the selected *Brucella* antigens by vaccinating mice with plasmid DNA (pCDNA, Invitrogen – fig. 7)

containing the specific *Brucella* genes under an eukaryotic promoter (cytomegaloviral promoter) and other essential transcriptional signal sequences. Currently we have immunized mice with DNA vaccines of GroEL using a variety of injection routes. Initial analysis indicated that all the immunized mice developed a strong antibody response against the specific proteins (fig. 7). CMI assays are being carried out. Mice in this pilot study were challenged with virulent *Brucella* to assess protection; no protection was obtained.

Figure 7.



A and B Antigen : RB51
A: Sera from mice immunized with DNA vaccine
B: Sera from mice immunized with RB51

We are initially targeting the GroEL, GroES, and SOD proteins since homologues of these proteins from other intracellular pathogens have been shown to be protective if a suitable antigen delivery system is used (15, 16, 17). It has been clearly demonstrated that delivery of appropriate antigens through DNA vaccination results in immune responses necessary for protection against several viral as well as bacterial pathogens (18,19).

E. Publications resulting from the contract support, personnel list and graduate degrees.

Toth, T.E., J.A. Cobb, S.M. Boyle, R.M. Roop and G.G. Schurig. Selective humoral immune response of Balb/C mice to *Brucella abortus* proteins expressed by vaccinia virus recombinants. Vet. Microbiol. 171-183, 1995.

Thesis: Intracellular growth of *Brucella abortus* and *B. melitensis* in murine macrophage-like cell lines and partial characterization of a biologically active extract from B. abortus strain RB51. Darla J. Wise. Ph.D, Virginia Tech, July 1997.*

Thesis: Assessment of the expression of *Brucella abortus* heat shock protein, GroEL, in vaccinia virus to induce protection against a Brucella challenge in

BALB/c mice. Simge Bologlu, Master of Sciences, Virginia Tech, June 1997.**

Vemulapalli R, Duncan AJ, Boyle SM, Toth TE, Sriranganathan N, Schurig GG. Isolation and expressional cloning of *secD* and *yajC* genes of *Brucella abortus*. Abstract# B-106, 97th General meeting, American Society for Microbiology, Miami Beach May 4-8, 1997.

Cravero S, Vemulapalli R, Toth TE, Calvert CL, Boyle SM, Sriranganathan N, Rosetti O, Schurig GG. Specific immune responses of BALB/c mice inoculated with recombinant vaccinia virus expressing *B. abortus* 18 kDa outer membrane protein. Abstract# E-73, 97th General meeting, American Society for Microbiology, Miami Beach May 4-8, 1997.

Vemulapalli R, Lopez-Santiago R, Moreno-Lafont M, Toth TE, Sriranganathan N, Boyle SM, Schurig GG. Characterization of immune responses of mice vaccinated with vaccinia virus expressing 18 kDa antigen of *Brucella abortus*. Brucellosis conference 1997.

Vemulapalli, R., A.J. Duncan, T.E. Toth, S.M. Boyle, N. Sriranganathan, and G.G. Schurig. Isolation of secD and yajC genes of *Brucella abortus* and vaccinia virus mediated expression of YajC. 2nd National Symposium Basic Aspects of Vaccines, Bethesda, MD, April/May 1997, Poster # B-2.

1996 A.J. Duncan, S.M. Boyle, T.E. Toth, N. Sriranganathan, and G.G. Schurig. *Brucella abortus* 2308 contains the genes encoding membrane transport proteins secD and yajC. CRWAD, Nov. 96, Chicago, Poster # P5

1998. Vemulapalli ,R., A. J. Duncan, S. M. Boyle, N. Sriranganathan, T.E. Toth, and G. G. Schurig* Cloning and Sequencing of *yajC* and *secD* Homologs of *Brucella abortus* and Demonstration of Immune Responses to YajC in Mice Vaccinated with *B. abortus* Strain RB51. In press. Infect. Immun.

Eid, H[1,2]., Y. Zhang[1], S. M. Boyle[1], **G.G. Schurig[1]**, M.E. Enany[2], M.E.El-Sayed[1,2] and N. Sriranganathan[1]. "Cloning and sequencing of a hypothetical transcriptional regulator gene from *Brucella melitensis* biotype 3." 50th Brucellosis Research Conference. Nov,8&9th, 1997 Chicago, IL.

The following individuals worked on this project and received pay from this contract as salary with benefits or hourly wages: J.Bard, D.Coker, L.Buccolo, R.Vemulapalli, C.Perdue, R.L.Speed, M.Knieriem, G.Machin-Scarpaci, M.Fallon, P.Dillon-Long,R.Jack, A.Zeytun,L.Colby, J.Duncan, C.Calvert, R.Rice, E.Hughes,C.Tilton-Lewis

* A Ph.D. degree was obtained by Mrs. Darla Wise; this project contributed funds for materials and supply for one section of her work related to the sequencing and attempts to locate the genes for the 10.2 and 11.6 kDa Brucella proteins. Stipends and other cost were not covered by this project.
** A MS degree was obtained by Mrs. Simge Bologlu; this project contributed funds for material and supplies related to the construction of vaccinia/Brucella recombinants and testing *in vivo* and *in vitro.* Stipends and other cost were not covered by this project.

VII. Conclusions.

During this period we have been able to discover several additional *Brucella* antigens potentially involved in a protective immune response as indicated by *in vitro* correlates of CMI like the induction of IgG2a antibodies, stimulation of lymphocyte proliferation and induction of INF-γ production. Some of these "antigens" may be virulence factors. The antigens can be purified in mg quantities as fusion proteins allowing for studies leading to better characterization and understanding of the immune response to these putative protective antigens. An alternate method of producing specific *Brucella* antigens using recombinant Baculovirus, also appears useful for such studies. Studies with pure antigens are important to insure that we are dealing with antigens that will induce appropriate immune responses.

Studies with some of our vaccinia/*Brucella* recombinants expressing these putative protective antigens constructed in our laboratory clearly indicate that vaccinia/Brucella recombinants can express *Brucella* antigens and that mice immunized with the recombinants are able to respond with an immune response against these antigens. Nevertheless, protection has not yet been induced with any of our constructs indicating that additional research is needed to optimize the system in order to induce protective immunity.

One possible reason for the protective failure is that the promoters selected to achieve expression of the putatively protective *Brucella* antigens are not strong enough and not enough *Brucella* antigen is produced during the virus replication cycle. New constructs are being made using early/late synthetic promoters to overcome this problem but, results are starting to suggest that the use of stronger synthetic, early/late promoters does not resolve the problem by itself.

A second possible reason is that the vaccinia system as applied in this project, may not be appropriate to induce the correct CMI responses needed for protection. This area is being explored by constructing vaccinia recombinants expressing listeriolysin. These recombinants should protect against challenge with *L. monocytogenes.* Depending on the results obtained, we may have to

further alter the vaccinia expression system to achieve protection. For example the use of vaccinia vectors expressing IL-12 may be necessary to achieve a strong TH1, protective immune response.

A third possible reason is that the antigens selected to induce a protective immune response are not protective antigens. This area is being explored by constructing vaccinia/*Brucella* L7/L12 recombinants. The L7/L12 antigen has been demonstrated to induce a certain level of protection therefore, vaccinia/L7/L12 recombinants should induce a protective immune response. It is probably incorrect to assume that *Brucella* antigens that demonstrate in vitro reactivities that correlate with in vivo induction of CMI are good candidates as protective antigens. This is exemplified by the 18kDa *Brucella* antigen that induces INF-γ responses and IgG2a production but is not a protective antigen. Therefore, these assays are probably not enough to conclude that the selected *Brucella* antigens do play a role in protection. Additional assays should be added to the *in vitro* panel ; we have initiated the use of cytotoxicity assay using sensitized lymphocytes. In addition, disruption mutants of putative protective *Brucella* antigens should be made from *B. abortus* strain RB51 and DNA vaccines should be prepared with the genes of the putatively protective antigens. These preparations can be tested for their ability to induce protection. After all these data are available a final selection of antigens of putative protective potential should be made for cloning into desired vectors. We were successful in producing both (disruption mutants and DNA vaccines) and they are being tested in mice for their ability to induce CMI and protection against challenge. Loss of protection using specific RB51 disruption mutants or protection afforded with DNA vaccines would strongly suggest that our selected antigens are of protective nature.

VIII. REFERENCES.

1.Schurig, G.G., R.M. Roop, T. Bagchi, D. Buhrman, S.M. Boyle, and N. Sriranganathan. 1991. Biological properties of RB5I: a stable rough strain of Brucella abortus. Vet. Microbiol 28:171-188.

2.Enright,F.M., J. Walker, G.G. Schurig, D. Buhrman. 1990. Brucella abortus RB5I: A better bovine Brucellosis vaccine ADRWSS, March 1990. Raleigh, NC abstract #16.

3. Cheville,N.F., M.G. Stevens, S.M. Halling,F.M. Tatum, A.E. Jensen. 1992. Protection against infection and abortion of cattle vaccinated with mutant strains of Brucella abortus. 73rd CRWAD, Nov. 9 -10, Chicago Il., abstract #42

4. Bagchi, T. 1990. Immune mechanisms in murine Brucellosis: Studies with strain RB5I, a rough mutant of Brucella abortus. Ph.D. Thesis, VPI & SU, Blacksburg, VA.

5. Bagchi,T. G.G. Schurig. 1990. Protection against Brucella abortus smooth strain by adoptive transfer of rough strain sensitized splenic lymphocytes. ADRWSS, Raleigh, NC abstract #3

6. Cheers, C. 1984. Pathogenesis and cellular immunity in experimental murine brucellosis. Dev. Biol. Stand. 56:237-246.

7. Araya, L.N.,P.H. Pelzer,G.E. Rowe,F.M. Enright,A.J. Winter 1989. Temporal development of protective cell mediated and humoral immunity in BALB/c mice infected with Brucella abortus. J. Immunol. 143:3330-3337.

8. Araya,L.N.,A.J. Winter. 1990. Comparative protection of mice against virulent and attenuated strains of Brucella abortus by passive transfer of immune T cells or serum. Infect. Immun. 58:254-256.

9. Mackaness, G.B. 1964. The immunological basis of acquired cellular resistance. J. Exp. Med. 120:105.

10. Halliburton, B. L. , R .D. Hindsdell. 1972. Recall of acquired resistance in mice by antigens from killed Brucella. Infect. Immun. 5:42-49.

11. Nash, T.W., D.M. Libby, M.A. Horwitz. 1988. IFN-g activated human alveolar macrophages inhibit the intracellular multiplication of Legionella pneumophilia. J. Immunol. 140:3978-3984.

12 . Belosevic,M., C.E. Davis, M.S. Meltzer, C.A. Nacy. 1988. Regulation of activated macrophage antimicrobial activities: Identification of lymphokines that cooperate with INF-g for induction of resistance to infection. J. Immunol. 141:890- 896.

13. Hahn,H.,S.H.E. Kaufmann. 1981. Role of cell mediated immunity in bacterial infections. Rev. Infec. Dis. 3:1221-1225.

14. Flesch,I., S.H.E. Kaufmann. 1987. Micobaterial growth inhibition by interferon-g activated bone marrow macrophages and differential susceptibility among strains of Mycobacterium tuberculosis. J. Immunol. 138:4408-4415.

15.Tascon RE, Colston MJ, Ragno S, Stavropoulos E, Gregory D, Lowrie DB. 1996. Vaccination against tuberculosis by DNA injection. Nat Med 2, 888-892.

16.Hess J, Dietrich G, Gentschev I, Miko D, Goebel W, Kaufmann SH. 1997. Protection against murine listeriosis by an attenuated recombinant *Salmonella typhimurium* vaccine strain that secretes the naturally somatic antigen superoxide dismutase. Infect Immun 65, 1286-1292 .

17.Ferrero RL, Thiberge JM, Kansau I, Wuscher N, Huerre M, and Labigne A. 1995. The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. Proc Natl Acad Sci USA 92, 6499-6503.

18.Donnelly, J.J., Ulmer, J.B., Liu, M.A. 1994. Immunization with DNA. J. Immunol. Methods 176, 145-152.

19. Hassett, D.E., Whitton, J.L. 1996. DNA immunization. Trends in Microbiol. 4, 307-312.

20. Lipford GB, Lehn N, Bauer S, Heeg K, Wagner H. 1994. The immunodominant peptide from listeriolysin in Quil A liposomes vaccinates CD8+ cytolytic T cells and confers protection to infection. Immunol Lett .May :40(2):101-104.

21. Mengaud J, Vivente MF, Chenevert J, et al. 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect Immun 56, 766-772.

23. Hemmen,F., V. Weynants, T. Scarcez, J-J Letesson and E. Saman. 1995. Cloning and sequence analysis of a newly identified Brucella abortus gene and serological evaluation of the 17-kilodalton antigen that it encodes. Clin. Diagn. Lab. Immunol.2: 263-267.

24. Toth, T.E., J.A. Cobb, S.M. Boyle, R.M. Roop and G.G. Schurig. Selective humoral immune response of Balb/C mice to *Brucella abortus* proteins expressed by vaccinia virus recombinants. Vet. Microbiol. 171-183, 1995.